Human Papillomavirus Infection and Esophageal Squamous Cell Carcinoma: A Case–Control Study

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Abstract

Background: The risk factors for esophageal squamous cell carcinoma (ESCC) in the high-incidence areas of China remain unclear.

Methods: A total of 300 patients with ESCC and 900 controls matched for age and sex were enrolled in Anyang (China), a high-risk area for ESCC in China. In tumor tissue of the cases and in esophageal biopsies of controls, the presence of human papillomavirus (HPV) DNA was assessed by an SPF1/GP6þ-mediated PCR followed by sequencing. The presence of serum antibody against the HPV-16 E7 oncoprotein was assessed by use of the ELISA. ORs with 95% confidence intervals (CI) were calculated via unconditional logistic regression models.

Results: The presence of HPV in the esophagus (OR, 6.4; 95% CI, 4.4–9.2) was associated with increased risk of ESCC. Moreover, infection with “oncogenic” types of HPV (OR, 10.3; 95% CI, 6.3–16.8) was more strongly associated with ESCC than other types of HPV (OR, 2.4; 95% CI, 1.4–4.2). The presence of HPV-16 (OR, 12.8; 95% CI, 7.6–21.7) was particularly strongly associated with ESCC. In addition, a higher proportion of cases than controls had serum antibodies against HPV-16 E7 (OR, 6.1; 95% CI, 3.7–10.0).

Conclusion and Impact: This study provides the strongest epidemiologic evidence to date in support of the important role of HPV in the development of ESCC in high-incidence areas of China.

Introduction

The incidence of squamous cell esophageal carcinoma (ESCC) varies considerably from place to place, suggesting an important role for environmental factors in its etiology (1). The etiologies of this disease seem to differ between low-incidence regions and high-incidence regions. For example, tobacco and alcohol use are important risk factors for esophageal cancer in industrial countries (2), whereas in high-incidence regions other factors, such as nutritional deficiency, have a stronger relation to disease incidence (3, 4). Northern China is one of the world’s highest incidence regions (5, 6), but studies that have been carried out there have not yet identified the basis for the high incidence (7–10).

Involvement of human papillomavirus (HPV) infection in esophageal cancer was first suggested in 1982 (11), but to date studies that have tested this hypothesis have not obtained consistent results (12–18). We have frequently detected HPV DNA in esophageal squamous cell carcinoma (ESCC) tissues collected from patients in Anyang (19), a high-incidence area for ESCC in China, whereas other studies failed to produce comparable results in samples collected from same region and concluded there was no association between HPV infection and ESCC (20–22). Our previous study (refer to the Supplementary data) and a study from other investigators have observed that HPV viral load in ESCC is much lower than in cervical cancer tissues (23). A low copy number of HPV in samples has been reported to generate discordant and false negative results (24–26). Therefore, low-HPV viral load in ESCC and different methods applied in these studies may account for the discrepancy among studies.

In this report, we conducted a case–control study with subjects from Anyang, China to evaluate HPV infection as a risk factor for the development of ESCC.
Methods

Subjects and specimen collection:
From December 2007 to September 2008, we recruited 300 consecutive newly diagnosed patients with ESCC who accepted esophagectomy from Anyang Cancer Hospital (Henan Province, PR China) for this study. Necrotic tumor specimens and serum were collected. Demographic data and personal information including age, sex, place of residence, tobacco and alcohol consumption history, and family history (immediate blood relatives within 3 generations) of esophageal cancer were obtained from patient medical records.

The controls were randomly selected from participants in a cohort study carried out among a representative sample of Anyang residents over the same time period. (27, 28) Three controls of the same gender and within the same 5-year age category were matched to each case. Esophageal tissues of controls were collected by gastro-endoscopic biopsy from a standard site in the midesophagus (25-cm distal to the incisors in the 6 o’clock position) and serum samples were also collected from these persons. A questionnaire about information similar to that obtained from the medical records of the ESCC cases was completed for each control subject in one-on-one interviews.

Esophageal tissue specimens from ESCC cases and normal controls were stored in −70°C freezers immediately after collection and processed in an identical manner. Rigorous quality control procedures were used to prevent contamination (27).

An individual informed consent was signed by all participants. This study was approved by the Institutional Review Board of the School of Oncology, Peking University, Beijing, PR China.

Laboratory study

DNA purification, HPV DNA detection, and typing.
DNA from tumor tissues and normal specimens were purified on a Biomek 3000 automated workstation by the E.Z.N.A.TM Mag-Bind Tissue DNA Kit (Omega Bio-Tek, Inc.). β-Globin was detected in all purified DNA samples. The samples were analyzed for the presence of HPV DNA by PCR, using a SPF1/GP6+ primer set which amplifies a fragment of approximately 184 bp in the L1 frame which has been shown to be highly sensitive (29). The HPV types were identified either by direct sequencing of PCR products or by cloning of PCR products and sequencing. Rigorous quality control procedures were used to avoid false positives as previously described (27).

Serologic analysis. Serum antibody against the HPV-16 E7 oncoprotein was detected with the ELISA, using bacterial expressed GST full-length E7 as the antigen (30). Mean ± 3 SD of OD929 in negative controls from the same plate was adopted as the cutoff value for defining positive ELISA results in this study. Twenty negative controls and 70 studied specimens were placed in one plate and the mean value of all the cutoffs was 0.080 (range, 0.074–0.086). All samples were tested for 3 times, and specimens which were positive at least 2 times were finally considered as positive.

Statistical analysis
In this study, we defined regular cigarette smoking as a history of at least 1 cigarette per day for ≥12 months or ≥18 packs for 1 year, and regular alcohol consumption was defined as drinking Chinese liquor at least twice per week for ≥12 months (other kinds of regular drinker such as beer and red wine is very rare in local area).

Differences in demographic characteristics and detection proportions of HPV DNA and HPV-16 E7 antibody in case and control groups were evaluated using the χ² test. Both unconditional and conditional logistic regression analyses were carried out to estimate ORs and 95% confidence intervals (CI) and only results from the unconditional models are presented, as the results from unconditional and conditional logistic models were very similar. Oncogenic and nononcogenic HPV were classified on the basis of the criteria in cervical cancer (31).

Statistical analyses were conducted using Stata 10.0 (StataCorp LP) for Windows, and all p values were 2 sided.

Results

Table 1 shows the demographic characteristics of the case and control groups and the presence of HPV DNA

| Table 1. Demographic characteristics and presence of HPV DNA and HPV-16 E7 antibody in cases and controls |
|---|---|---|
| Age, y | Case (N = 300) | Control (N = 900) | P<sup>a</sup> |
| 30–40 | 4 (1.3) | 14 (1.6) | — |
| 41–50 | 27 (9.0) | 96 (10.7) | — |
| 51–60 | 166 (55.3) | 522 (58.0) | — |
| 61–70 | 103 (34.3) | 268 (29.8) | — |
| Gender | | | |
| Male | 178 (59.3) | 534 (59.3) | — |
| Female | 122 (40.7) | 366 (40.7) | — |
| History of regular alcohol consumption | | | |
| No | 252 (84.0) | 745 (82.8) | 0.625 |
| Yes | 48 (16.0) | 155 (17.2) | 0.128 |
| History of regular cigarette smoking | | | |
| No | 163 (54.3) | 518 (57.6) | 0.329 |
| Yes | 137 (45.7) | 382 (42.4) | <0.001 |
| Family history of ESCC | | | |
| HPV DNA positive | 93 (31.0) | 61 (6.8) | <0.001 |
| HPV-16 E7 antibody positive | 51 (17.0) | 28 (3.1) | <0.001 |

<sup>a</sup>P values derived from the χ² test.
Table 2. Type-specific detection of HPV infection in esophagus in ESCC cases and controls

<table>
<thead>
<tr>
<th>Types</th>
<th>Genus</th>
<th>Species</th>
<th>Case (N = 300)</th>
<th>Control (N = 900)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oncogenic type</td>
<td></td>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>HPV-16</td>
<td>Alpha</td>
<td>9</td>
<td>72 (24.0)</td>
<td>27 (3.0)</td>
</tr>
<tr>
<td>HPV-18</td>
<td>Alpha</td>
<td>7</td>
<td>2 (0.7)</td>
<td>5 (0.6)</td>
</tr>
<tr>
<td>HPV-58</td>
<td>Alpha</td>
<td>9</td>
<td>0 (0.0)</td>
<td>1 (0.1)</td>
</tr>
<tr>
<td>&quot;Nononcogenic&quot; type</td>
<td></td>
<td></td>
<td>27 (8.0)</td>
<td>35 (3.9)</td>
</tr>
<tr>
<td>HPV-57</td>
<td>Alpha</td>
<td>4</td>
<td>15 (5.0)</td>
<td>7 (0.8)</td>
</tr>
<tr>
<td>HPV-3</td>
<td>Alpha</td>
<td>2</td>
<td>9 (3.0)</td>
<td>24 (2.7)</td>
</tr>
<tr>
<td>HPV-94</td>
<td>Alpha</td>
<td>2</td>
<td>2 (0.7)</td>
<td>4 (0.4)</td>
</tr>
<tr>
<td>HPV-27</td>
<td>Alpha</td>
<td>4</td>
<td>1 (0.3)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

*The total number of type-specific positives was larger than the total number of HPV positives because of multiple infections, namely, 2 infections with HPV-16 and HPV-18, 3 coinfections with HPV-16 and HPV-57, and 2 coinfections with HPV-16 and HPV-3.

Oncogenic and nononcogenic types were defined according to their carcinogenicity in cervical cancer (31, 36).

and HPV-16 E7 antibody in the 2 groups. Case and control subjects were similar in alcohol consumption and cigarette smoking. Compared with the control group, there was a higher prevalence of HPV DNA (31.0% vs. 6.8%, P < 0.001) and HPV-16 E7 antibody in the serum (17.0% vs. 3.1%, P < 0.001) of cases. Also, a family history of esophageal cancer (24.0% vs. 11.9%, P < 0.001) was found more frequently in the case group.

As shown in Table 2, a total of 7 HPV genotypes were identified in ESCC tumor tissue and normal esophageal tissue in our sample, including HPV-16, 18, 58, 57, 3, 94, and 27, all of which belong to the genus alpha. Multiple infections were found in 6 case patients and 1 control, including 3 coinfections with HPV-16 and 57, 2 coinfections with HPV-16 and 3, and 2 coinfections with HPV-16 and 18. On the basis of the criteria which have been used in cervical cancer, we categorized the HPV types into oncogenic and nononcogenic types (31). HPV-16 (23.3% in cases vs. 2.3% in controls) was the predominant type among the oncogenic types, HPV-57 (5.0%) was the predominant nononcogenic type in cases, and HPV-3 (2.7%) was the predominant nononcogenic type in controls. The frequency of detection of oncogenic and nononcogenic types of HPV in the case group were both higher than in the control group (24.0% vs. 3.0%, 9.0% vs. 3.9%, P < 0.001).

Four unconditional logistic regression models were fitted as shown in Table 3 to evaluate the association between HPV infection and ESCC, in which, all of the 300 patients with ESCC and 900 normal controls were included. In model 1, detection of any type of HPV DNA (OR, 6.4; 95% CI, 4.4–9.2), detection of HPV-16 E7 antibody in serum (OR, 6.1; 95% CI, 3.7–10.0), and a family history of esophageal cancer (OR, 2.5; 95% CI, 1.8–3.6) were strongly and positively associated with ESCC. However, there was no evidence of a relationship between consumption of alcohol (OR, 0.9; 95% CI, 0.6–1.4) or tobacco (OR, 1.2; 95% CI, 0.8–1.7) and ESCC. In models 2, 3, and 4, the associations between nononcogenic, oncogenic HPV, and HPV-16 infections and ESCC were assessed. Compared with model 1, a higher OR (OR, 10.3; 95% CI, 6.3–16.8) for oncogenic type HPV and a relatively lower but still elevated OR (OR, 2.4; 95% CI, 1.4–4.2) for nononcogenic type HPV infection were observed. When HPV-16 infection was analyzed separately, the relationship between HPV-16 and ESCC (OR, 12.8; 95% CI, 7.6–21.7) was particularly strong.

Discussion

On the basis of the discovery of HPV-associated koilocytes in esophageal squamous cell papillomas and esophageal carcinomas, involvement of HPV infection in esophageal cancer was suggested as early as 1982 (11). However, the presence of HPV DNA in esophageal carcinomas has been a highly controversial issue (16, 20, 22, 32). Differences in sampling methods, demographic and ethnic factors, disease status, and sensitivity of detection methods have been suggested to be potential causes of the discrepancies in findings (16). Variation in the frequency of detection using identical samples and methodology were also observed in our own laboratory, prompting us to investigate the reason behind this variation. Real-time PCR revealed that the HPV copy number in esophageal cancer specimens was at least 2 orders of magnitude lower than that in cervical cancer. This low HPV copy number seems to have been a cause of poor intralaboratory reproducibility and has the potential to result in a falsely low level of detection in ESCC. On this basis, we established an analytic strategy for HPV detection in esophageal cancers as previously described (19). Briefly, L1 detection was carried out twice for each esophageal sample followed by sequencing or cloning and sequencing of the PCR product. L1 positivity which was confirmed by sequencing in either test was counted as positive for calculation of the detection proportion. In addition, the SPF1/GP6+ primer set gives a higher amplification efficiency than GF5+/6+.
Table 3. Unconditional logistic regression analysis of the association between HPV infection and ESCC

<table>
<thead>
<tr>
<th>Variables</th>
<th>Model 1 (any type)</th>
<th>Model 2 (oncogenic type)</th>
<th>Model 3 (nononcogenic type)</th>
<th>Model 4 (HPV-16)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude OR (95% CI)</td>
<td>Adjusted OR (95% CI)</td>
<td>Crude OR (95% CI)</td>
<td>Adjusted OR (95% CI)</td>
</tr>
<tr>
<td>HPV DNA in esophageal tissue</td>
<td>207/839 1.0</td>
<td>229/784 1.0</td>
<td>273/855 1.0</td>
<td>228/793 1.0</td>
</tr>
<tr>
<td>Positive</td>
<td>93/61 6.2 (4.3–8.8)</td>
<td>6.4 (4.4–9.2)</td>
<td>71/26 3.8 (2.4–6.2)</td>
<td>7/21 4.7 (2.5–8.9)</td>
</tr>
<tr>
<td>Negative</td>
<td>98/373 1.0</td>
<td>10.4 (6.5–16.7)</td>
<td>27/35 10.0 (4.1–25.3)</td>
<td>7/21 12.8 (7.7–21.7)</td>
</tr>
<tr>
<td>HPV-16 E7 antibody in serum</td>
<td>249/792 1.0</td>
<td>249/792 1.0</td>
<td>51/26 6.4 (3.9–9.9)</td>
<td>7/21 12.7 (7.7–21.7)</td>
</tr>
<tr>
<td>Positive</td>
<td>51/28 6.4 (3.9–10.3)</td>
<td>6.2 (3.9–10.3)</td>
<td>51/26 6.4 (3.9–10.3)</td>
<td>7/21 12.7 (7.7–21.7)</td>
</tr>
<tr>
<td>Negative</td>
<td>249/792 1.0</td>
<td>10.5 (4.4–25.3)</td>
<td>51/26 6.4 (3.9–10.3)</td>
<td>7/21 12.7 (7.7–21.7)</td>
</tr>
<tr>
<td>Family history of ESCC</td>
<td>228/793 1.0</td>
<td>228/793 1.0</td>
<td>51/26 6.4 (3.9–10.3)</td>
<td>7/21 12.7 (7.7–21.7)</td>
</tr>
<tr>
<td>No</td>
<td>12/107 2.3 (1.4–3.7)</td>
<td>2.3 (1.4–3.7)</td>
<td>51/26 6.4 (3.9–10.3)</td>
<td>7/21 12.7 (7.7–21.7)</td>
</tr>
<tr>
<td>Yes</td>
<td>72/107 2.3 (1.4–3.7)</td>
<td>2.3 (1.4–3.7)</td>
<td>51/26 6.4 (3.9–10.3)</td>
<td>7/21 12.7 (7.7–21.7)</td>
</tr>
</tbody>
</table>

*The oncogenic and nononcogenic type was defined according to their carcinogenicity in cervical cancer (31, 36).

bAdjusted ORs and 95% CIs derived from logistic models including all listed variables as well as age, gender, tobacco use, and alcohol consumption.

There are several limitations in this study. Because the nonlaboratory data for ESCC cases were obtained from patient medical records, information concerning some potential confounders was not included in the study, such as household income, educational level, dietary habits, and sexual behavior, some of which have been reported to be associated with HPV infection or ESCC in local area previously (8, 35). However, we believe that these factors and allows amplification of some novel viral types such as HPV-57. However, this primer set may decrease the amplification efficiency for HPV-18 (data not shown). Therefore, the possibility that some HPV-18 infections were missed in this study cannot be excluded.

In this study, we enrolled 300 ESCC cases from Anyang Cancer Hospital, which is located in a region in China of high-ESCC risk. More than 90% of the cases came from the rural areas of Anyang, and the 900 matched controls were randomly selected from a population-based cohort in the same area. The presence of HPV DNA in esophagus and the antibody against HPV-16 E7 oncoprotein in serum were compared between case and control groups. HPV DNA in esophagus reflects the current infection status, and serum antibody indicates the past and cumulative expression of E7 oncoprotein which is crucial for the carcinogenesis. After controlling for a series of potential confounding factors, we observed a strong association between HPV infection and ESCC, both on DNA level and in the serologic analysis.

A total of 7 genotypes of HPV, all of which belong to the genus alpha were detected in our sample, including 3 oncogenic types (HPV-16, 18, and 58) and 4 nononcogenic types (HPV-57, 3, 94, and 27). Oncogenic HPV had a stronger relationship with ESCC than did nononcogenic types. HPV-16 infection made the strongest contribution to the association between HPV infection and ESCC. In addition, the presence of serum antibody against HPV-16 E7 oncoprotein was a predictor of ESCC. A family history of esophageal cancer was associated with ESCC, which is consistent with previous studies in high-risk areas for ESCC in China (33, 34). However, the influence of family history by itself does not necessarily indicate a genetic role in the etiology of cancer, and exposure to common environmental factors could also explain the relationship.

One might expect that the seropositives of HPV-16 E7 fall within the HPV-16 DNA positives, but a fairly poor concordance between them was found in both genders ($k_{combined} = 0.130, P < 0.001; k_{male} = 0.16, P < 0.001; k_{female} = 0.09, P = 0.03$) in this study. Because the presence of HPV DNA and serum antibody reflect current infection in the esophagus and past exposure of HPV, respectively, and HPV infection often is eliminated within a short period of time, only a prospective study with multiple cross-sections is likely to have the ability to detect their concordance. Nonetheless, we have observed that HPV-16 E7 seropositive ESCC cases in this study had a significantly higher frequency of HPV DNA detection than controls (21 of 51 vs. 0 of 28, $P < 0.001$) indicating that viral E7 expression may predispose to ESCC.

There are several limitations in this study. Because the nonlaboratory data for ESCC cases were obtained from patient medical records, information concerning some potential confounders was not included in the study, such as household income, educational level, dietary habits, and sexual behavior, some of which have been reported to be associated with HPV infection or ESCC in local area previously (8, 35). However, we believe that these factors...
would have limited influence on the conclusions of this study, because case and control groups seemed to be highly comparable demographically, and because of the strong and stable association between HPV infection and ESCC that was observed. In addition, the temporal sequence of HPV infection and onset of ESCC cannot be ascertained. Therefore, a conclusion about a causal relationship between exposure and outcome must remain a tentative one, despite the strong association of infection and tumor which has been observed. A prospective cohort study would be needed to further address this issue.

In conclusion, HPV infection in the esophagus and serum antibody to HPV-16 E7 oncoprotein were both associated with an increase in risk of ESCC. High-risk types of HPV, especially HPV-16, were most strongly associated, but infection with low-risk type HPV for cervical cancer also showed some association with ESCC. To our knowledge, this study provides the strongest epidemiologic evidence to date in support of an association between HPV infection and ESCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Writing, review, and/or revision of the manuscript: F. Guo, Y. Liu, Z. He, N.S. Weiss, M.M. Madeleine, H. Cai, Y. Ke

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